

Dean L. Engelhardt et al.

Serial No.: 10/713,183

Filed: November 14, 2003

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### **REMARKS**

In the claim listing above, claims 112, 143 and 146 have been amended. Claims 141 and 142 have been canceled. New claims 149 and 150 have been added. Accordingly, as amended above, claims 112-120, 123-130, 133-140 and 143-150 continue to be presented for further examination in this application.

#### **Changes to the Claims**

As just indicated, step (d) in claim 112 has been amended in two instances. First, step (d) now recites "digesting said substrate with RNase H to remove said ribonucleic acid segment of said extended primer, *wherein said removal allows* another primer binding event to occur." Thus, the two words "and allow" in step (d) have been replaced by the foregoing italicized words. Second, for the sake of clarity, step (d) in claim 112 now recites *said DNA molecule of interest* instead of "said nucleic acid of interest." See below the rejection under 35 U.S.C. §112, second paragraph.

Claims 143 and 146 have also been amended. In each of claims 143 and 146, step (d) has been amended and now recites "to render said primer binding site available for another primer binding event and *thereby* producing more than one copy of said DNA molecule."

In addition, claims 141-142 have been canceled in favor of new claims 149-150, the latter now depending from claims 143 and 149. See the claim objection below.

It is believed that the foregoing amendments are supported by Applicants' original disclosure and, therefore, constitute subject matter to which they are duly entitled to claim.

Entry of the above amendments and new claim listing is respectfully requested.

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### **Withdrawal of Previous Rejection**

Applicants appreciate the indication in the Office Communication (page 2) that the rejection under 35 U.S.C. §103(a) has been withdrawn, and that their Terminal Disclaimer filed on January 21, 2009 has been reviewed, accepted and recorded.

### **Claim Objections**

According to the Office Communication (page 3):

Claims 141-142 stand objected to because they depend from cancelled claims. The claims should be amended to depend from an amended claim or the claims should be amended to incorporate the cancelled subject matter. As such the claims have not been further treated on the record.

The objection to claims 141-142 is believed to have been obviated by the cancellation of both claims, and the addition of new claims 149-150, which depend from claims 143 and 149, respectively.

Withdrawal of the claim objections is respectfully requested in light of these claim changes.

### **The Rejection Under 35 U.S.C. §112, First Paragraph**

Claim 112-120 and 141-142 stand rejected for indefiniteness under 35 U.S.C. §112, second paragraph. According to the Office Communication (page 3):

Claims 112-120 are indefinite. Claim 112 recites the limitation "said nucleic acid of interest" in line 3 of step d. There is insufficient antecedent basis for this limitation in the claim. It is suggested that the claim be amended to e.g. said DNA molecule of interest.

Claims 141-142 are indefinite. MPEP 608.01 (n)[R-3](V) states that "If the base claim has been cancelled, a claim which is directly or indirectly dependent thereon should be rejected as incomplete". Herein the instant case Claims 141-142 are dependent upon Claim 91 which has been cancelled.

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As indicated above, claim 112 has been amended in accordance with the Examiner's suggestion in this rejection.

As also indicated above, claims 141-142 have been canceled in favor of new claims 149-150, the latter claims now depending properly from claims 143 and 149, respectively.

In view of the above claim amendments, Applicants respectfully request reconsideration and withdrawal of the indefiniteness rejection.

#### **The Rejection Under 35 U.S.C. §112, First Paragraph**

Claims 146-148 stand rejected under 35 U.S.C. 112, first paragraph, for failing to comply with the written description requirement. According to the Office Communication (page 4):

Claims 146-148 are rejected as failing to comply with the written description requirement. Upon review of the specification, the specification does not appear to provide support for the recitation of "a reverse transcriptase having RNase H activity" in Claim 146. In response to the amendments, applicants have not pointed to any particular teaching in the specification.

The instant specification provides support for an effective amount of a reverse transcriptase, but does not limit the reverse transcriptase to having RNase H activity. Schuster et al. (US Patent 5169766 December 8, 1992) teaches that reverse transcriptase can have RNase H activity or it can not have RNase H activity (Column 8 lines 17-24). The instant specification does not provide support for the narrower limitation of only reverse transcriptase with RNase H activity.

These amendments to the claims, therefore, constitute new matter.

The written description rejection is respectfully traversed.

In the new matter rejection, the Office Communication cites a lack of support for the expression "a reverse transcriptase having RNase H activity." Applicants respectfully point out, however, that in [002] of the corresponding published U.S. 2005/0123926, reference was made in [0009] to US Patent 5,130,238 (Incorporated by reference) that describes the concomitant use of RNase H and Reverse Transcriptase

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in conjunction with RNA polymerase for their system. The '238 Patent discloses that "RNase H is an intrinsic activity of AMV" and as such a Reverse Transcriptase with RNase H activity is referenced. It should be noted that all future citations from the specification will use the published application, US 2005/0123926, for reference points.

It should also be pointed out that at the time of the present invention, an enzyme that would be referred to simply as "reverse transcriptase" would ordinarily be understood to have RNase H activity unless it was specified as not having it. This is due to the fact that the presence of RNase H activity is a normal characteristic of such enzymes. Thus, in a recent review by Champoux and Schultz on RNase H "Ribonuclease H: properties, substrate specificity and roles in retroviral reverse transcription." (2009 FEBS Journal 276; 1506-1516), the first line of the abstract states:

Retroviral reverse transcriptases possess both a DNA polymerase  
and an RNase activity.

A copy of Champoux and Schuliz's FEBS publication is provided in Appendix A.

Reverse transcriptases lacking this activity are exceptions that have been artificially created by mutations or deletions to inactivate this particular activity. Indeed, the enzyme in the cited Schuster '766 Patent (Column 8, lines 17-24) is Superscript, a proprietary enzyme that was engineered to lack RNase H activity and which was considered to be sufficiently novel to receive US Patent No. 5,244,797. As such, unless it is described as "RNase H (-)," a Reverse Transcriptase can be assumed to have the native RNase H activity.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the written description rejection.

#### **The Rejection Under 35 U.S.C. §102**

Claims 112-120, 123-130, 143-145 stand rejected under 35 U.S.C. §102(b) as being anticipated by Scheele (US Patent 5,162,209, issued on November 10, 1992). According to the Office Communication (pages 5-7):

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With regard to Claim 112, step a, Scheele teaches providing a first DNA strand (e.g. a DNA molecule of interest) (column 3 lines 25).

With regard to step b, Scheele teaches contacting the DNA with dNTPs (e.g. nucleic acid precursors) (Column 4 lines 20-22). Scheele teaches a primer comprising an RNA segment (Column 3 lines 33-40). Scheele teaches a method of adding E. coli DNA polymerase I (e.g. effective amount of nucleic acid producing catalyst) (column 4 lines 14-15). Scheele teaches a method of adding RNase H (Column 4 lines 25).

With regard to step c, Scheele teaches a method of carrying out synthesis in the presence of the RNA primer to generate a polynucleotide comprising an RNA/DNA hybrid (Column 3 lines 30-40 and Figure 5).

With regard to step d, Scheele teaches a method of digesting the substrate with RNase H to remove the ribonucleic acid segment of the extended primer (Column 3 lines 30-40, column 4 lines 25-28, and Figure 5). Scheele teaches that the method can be adapted to permit amplification of the sample of dsDNA by PCR methodology (column 8 lines 58-60). Scheele teaches that the reagents of the RNA primer, DNA target, dNTPs and Taq are placed into a PCR machine with the appropriate number of PCR temperature cycles (column 8 lines 65-69). Therefore in a PCR cycle the resultant dsDNA of step c would be denatured such that the strands of DNA produced which are identical to the DNA of interest can be used in the PCR cycle to produce more copies of the DNA of interest.

With regard to Claim 113, Scheele teaches that the primers comprise unmodified nucleotides because Scheele teaches the primers comprise nucleotides (Figure 5 step 3).

With regard to Claim 114, Scheele teaches that the primer includes a portion with that is complementary to an oligonucleotide tail added to the 3' end of the target DNA template (column 3 lines 25-35). Therefore the primers include a sequence which is complementary to the tail and therefore not complementary to the sequence of the DNA molecule of interest (e.g. the tail is added to the DNA target of interest but the tail is not considered the DNA which is of interest to be amplified) (Figure 5 and column 3 lines 25-40).

With regard to Claim 115, Scheele teaches that the primers comprise at least 5 nucleotides (column 3 lines 56-57).

With regard to Claim 116, Scheele teaches that the primer can include DNA and RNA because Scheele teaches that only some of the nucleotides of the primer are RNA (Column 3 lines 40-45).

With regard to Claim 117, Scheele teaches that the nucleic acid producing catalyst is a DNA polymerase (column 4 lines 14-15).

With regard to Claim 118, Scheele teaches that the DNA

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polymerase is *E. coli* DNA polymerase I (Column 4 lines 14-15).

With regard to Claim 119, Scheele teaches that the polymerase can also include Taq polymerase (Column 8 lines 66).

With regard to Claim 120, Scheele teaches that the nucleic acid precursors can be labeled (column 8 lines 37-38).

With regard to Claim 123, step a, Scheele teaches providing a first DNA strand (e.g. a DNA molecule of interest) (column 3 lines 25).

With regard to step b, Scheele teaches contacting the DNA with dNTPs (e.g. nucleic acid precursors) (Column 4 lines 20-22). Scheele teaches a primer comprising an RNA segment and a DNA segment by teaching that some of the nucleotide sequence of the primer is an RNA sequence and therefore the rest of the nucleotides would be DNA (e.g. a copolymer primer) (Column 3 lines 33-40). Scheele teaches a method of adding *E. coli* DNA polymerase I (e.g. effective amount of nucleic acid producing catalyst) (column 4 lines 14-15). Scheele teaches a method of adding RNase H (Column 4 lines 25).

With regard to step c, Scheele teaches a method of carrying out synthesis in the presence of the RNA primer to generate a polynucleotide comprising an RNA/DNA hybrid (Column 3 lines 30-40 and Figure 5).

The Office Communication continues on pages 8-11:

With regard to step d, Scheele teaches a method of digesting the substrate with RNase H to remove the ribonucleic acid segment of the extended primer (Column 3 lines 30-40, column 4 lines 25-28, and Figure 5). Scheele teaches that the method can be adapted to permit amplification of the sample of dsDNA by PCR methodology (column 8 lines 58-60). Scheele teaches that the reagents of the RNA primer, DNA target, dNTPs and Taq are placed into a PCR machine with the appropriate number of PCR temperature cycles (column 8 lines 65-69). Therefore in a PCR cycle the resultant dsDNA of step c would be denatured such that the strands of DNA produced which are identical to the DNA of interest can be used in the PCR cycle to produce more copies of the DNA of interest.

Scheele teaches a method of adding excess primer (e.g. multiple copies of the copolymer primer) (Column 8 lines 58-60). Scheele et al. teaches that once the dsDNA is generated RNase H is used to remove the RNA primer (column 9 lines 1-5). Therefore once the RNA segment from the primer is removed the template is used to amplify another target strand by using another copolymer primer.

With regard to Claim 124, Scheele teaches that the primers comprise unmodified nucleotides because Scheele teaches the primers comprise nucleotides (Figure 5 step 3).

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With regard to Claim 125, Scheele teaches that the primer includes a portion that is complementary to an oligonucleotide tail added to the 3' end of the target DNA template (column 3 lines 25-35). Therefore the primers include a sequence which is complementary to the tail and therefore not complementary to the sequence of the DNA molecule of interest (Figure 5 and column 3 lines 25-40).

With regard to Claim 126, Scheele teaches that the primers comprise at least 5 nucleotides (column 3 lines 56-57).

With regard to Claim 127, Scheele teaches that the nucleic acid producing catalyst is a DNA polymerase (column 4 lines 14-15).

With regard to Claim 128, Scheele teaches that the DNA polymerase is E. coli DNA polymerase I (Column 4 lines 14-15).

With regard to Claim 129, Scheele teaches that the polymerase can also include Taq polymerase (Column 8 lines 66).

With regard to Claim 130, Scheele teaches that the nucleic acid precursors can be labeled (column 8 lines 37-38).

With regard to Claim 143, step a, Scheele teaches providing a first DNA strand (e.g. a DNA molecule of interest) (column 3 lines 25).

With regard to step b, Scheele teaches contacting the DNA with dNTPs (e.g. nucleic acid precursors) (Column 4 lines 20-22). Scheele teaches a primer comprising an RNA segment and a DNA segment by teaching that some of the nucleotide sequence of the primer is an RNA sequence therefore the primer sequence would include DNA (e.g. a copolymer primer) (Column 3 lines 33-40). Scheele teaches a method of adding E. coli DNA polymerase I (e.g. effective amount of nucleic acid producing catalyst) (column 4 lines 14-15). Scheele teaches a method of adding RNase H (Column 4 lines 25).

With regard to step c, Scheele teaches a method of carrying out synthesis in the presence of the RNA primer to generate a polynucleotide comprising an RNNDNA hybrid (Column 3 lines 30-40 and Figure 5). The instant specification does not define isostatic conditions of temperature, buffer and ionic strength. Scheele teaches combining the primer and the DNA molecule in a reagent solution at a particular heat with buffers that have a particular ionic strength to produce at least one copy of the DNA molecule by extension of the primer (Column 8 lines 1-15). Therefore Scheele teaches a method of allowing the mixture to react under isostatic condition of temperature, buffer, and ionic strength.

With regard to step d, Scheele teaches a method of digesting the substrate with RNase H to remove the ribonucleic acid segment of the extended primer (Column 3 lines 30-40, column 4 lines 25-28, and Figure 5). Scheele teaches that the method can be adapted to permit amplification of the sample of dsDNA by PCR methodology (column 8

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lines 58-60). Scheele teaches that the reagents of the RNA primer, DNA target, dNTPs and Taq are placed into a PCR machine with the appropriate number of PCR temperature cycles (column 8 lines 65-69). Therefore in a PCR cycle the resultant dsDNA of step c would be denatured such that multiple copies of the DNA could be copied from the DNA of interest.

With regard to Claim 144, Scheele teaches that the primer can include DNA and RNA (Column 3 lines 40-45).

With regard to Claim 145, Scheele teaches that the primer includes a portion which is complementary to an oligonucleotide tail added to the 3' end of the target DNA template (column 3 lines 25-35). Therefore the primers include a sequence which is complementary to the tail and therefore not complementary to the sequence of the DNA molecule of interest (e.g. the tail is added to the DNA target of interest) (Figure 5 and column 3 lines 25-40).

The anticipation rejection is respectfully traversed.

In response and at the outset, Applicants respectfully note that Scheele is described as providing a DNA molecule of interest and also as teaching a primer comprising an RNA segment. It should be noted, however, that the method described by Scheele intrinsically requires an additional step, where the segment of the DNA where a primer will bind is artificially added onto the DNA molecule by a ligation step or Terminal Transferase extension. Such an obligatory step is missing in Applicants' invention and claim 112. An important distinction between the present invention and the Scheele's method can also be seen in the latter's Figure 5, where RNase H digestion is used to remove an RNA primer. In contrast to the present invention, the newly exposed single-stranded region in Scheele's disclosure is not used for binding of another RNA primer; rather, it is digested with a single-strand specific exonuclease thereby preventing any subsequent primer binding events. This illustration is also described in Scheele's text:

The next step is to remove the homopolymeric primer and tail. The Oligo(rG)<sub>15</sub> primer, which at this point comprises the 5' end of the second DNA strand, is removed from the duplex molecule by treatment with the enzyme RNase H; simultaneously the poly (dC) tail is removed by the 3'-5' exonucleolytic activity of T4 RNA polymerase (Column 7, lines 61-67).

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As such, there is no description in Scheele for preserving the single-stranded segment that is generated by treatment of an RNA primer with RNase H so that more binding and extension events can take place to generate more copies of the nucleic acid of interest. Furthermore, the illustrative example presented by Scheele (Column 7, line 16-Column 8, line 42) would not even allow such events to take place for several reasons. First, as noted above, the exonuclease is present at the same time as the RNase H, thereby eliminating the primer binding site that would be needed for binding of a second RNA primer. Second, the step before the RNase H step entailed inactivation of Pol I which would render the polymerase incapable of using the RNA primer to make a second copy by an extension reaction. With regard to the last exception, it should be noted that in column 2, [0019] of the '926 application the statement is made:

The regeneration of a primer binding site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

A priming event would be considered to be both the binding of a primer to its complementary site as well as extension (i.e. the primer acting as a primer) and consequently, a second priming event results in synthesis of a second copy. Therefore, when Scheele describes the use of an RNA primer, he not only does not teach the present invention, he actually and actively teaches away from it, by purposefully carrying out a step that prevents second binding events after RNase H digestion.

Page 5 of the Office Communication also expressed the opinion that with regard to step (d), PCR can be "adapted" to make more copies. Step (d) of the present invention and claim 112 stipulates that the RNase H removal of an RNA segment of an extended primer is what allows another primer binding and production of more copies whereas PCR uses thermal denaturation to allow additional primer binding events. To avoid any possible misinterpretations, Applicants have amended step (d) of claim 112 to read "digesting said substrate with RNase H to remove said ribonucleic acid segment of said extended primer, *wherein said removal allows* another priming event to occur

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with said nucleic acid of interest, thereby producing multiple copies of said nucleic acids of interest." The foregoing amendment to claim 112 should add more clarity to the concept that it is the digestion that allows another priming event to take place by a primer binding site. It should be noted that in reference to the use of PCR on page 6, the Office Communication states:

Therefore in a PCR cycle the resultant ds DNA of step c would be denatured such that the strands of DNA produced which are identical to the DNA of interest can be used in the PCR cycle to produce more copies of the DNA of interest.

It can be clearly seen that there is no description of a denaturation step after step c in the steps recited for claim 112 and in the PCR-centric system described in the Office Communication, digestion with RNase H has no participation in generation of more copies of the nucleic acid of interest. Thus, the Office Communication has substituted a completely different step (d) to replace the one actually described in present claim 112.

With reference to page 6, it should be pointed out that claim 114 is dependent from claim 112. If the Office Communication now desires to consider the sequences of the Scheele primers to be complementary to the tail but "not complementary to the sequence of the DNA molecule of interest", the Scheele primers no longer suit the definition required by present claim 112, which requires a primer comprising a sequence complementary to a sequence of the DNA molecule of interest. Claim 114 actually is a claim that has an additional limitation where the primer is now required to have two segments, a first segment that is complementary to a sequence in the DNA interest (to fulfill the conditions of claim 112) and a second segment comprising additional sequences that are not complementary (to fulfill the added limitation of claim 114). The homopolymeric primers of Scheele cannot be stretched by any reasonable interpretation to encompass sequences that are both complementary and non-complementary at the same time. The Office Communication is taking the mutually

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exclusive positions that the definition of the "DNA molecule of interest" comprises the additional segment in claim 112 but lacks this segment in claim 114.

With reference to page 7 and comments on claim 123, the same remarks concerning claim 112 are maintained, i.e., there is no suggestion in Scheele of an RNase H step allowing the binding and extension of a second RNA primer. On page 8, there is a more complete description of the adaptation of PCR to Scheele's method but as noted previously, allowing a series of primers binding to the same primer binding site is now being carried out by a denaturation step rather than the RNase H step required by the present invention and claims. The description of the process by Scheele reads as follows:

... to a sample of ds cDNA prepared by the method of the invention, with its RNA primer/DNA extension still intact, is added excess RNA primer (identical to the RNA primer used to generate the original ds cDNA) and excess oligo (dT) primer..... the mixture is subjected to an appropriate number of PCR cycles in a PCR machine...." (Column 8, lines 60-68).

It can be clearly seen that in this alternative methodology, the use of RNase H has been eliminated, and primer removal is now carried out by the thermal cycling of PCR. An RNase H step is only added by Scheele after amplification in conjunction with exonuclease (Column 9, lines 1-5) and it is not responsible itself for any amplification, only for "trimming" the PCR product. Only thermal denaturation is described as allowing the use of a target strand by another copolymer primer in Scheele, whereas, the language as stated in present claim 123 makes it abundantly clear that the removal of the RNA segment is what allows another copolymer to initiate synthesis in the present invention. Consequently, Applicants also take issue with the conclusory statement on page 8 of the Office Communication:

Therefore once the RNA segment from the primer is removed the template is used to amplify another target strand by using another polymer

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since it can be seen that in the context of the section cited from Scheele, the statement of the Office Communication is the temporal reverse of the actual situation since Scheele actually taught that once a target strand has been amplified by another polymer, the RNA segment from the primer is removed. There is no description in Scheele's patent of any amplification taking place after the RNase H step.

With reference to page 10, and claim 143, the statement is made in the Office Communication that

The instant specification does not define isostatic conditions of temperature, buffer, and ionic strength.

Applicants are uncertain of whether the Office Communication has apprehensions concerning the meaning of "isostatic" or for a lack of a recitation of the particular conditions that would be maintained on an isostatic basis. With regard to the former, the conditions are essentially self-defining as isostatic is given the ordinary meaning of "not changing", thus it would be akin to the commonly used term "isothermal" with the added proviso that it also encompasses no changes in buffer and ionic strength during the reaction as well. With regard to the latter, the instant application may not describe what the exact parameters would be for temperature, buffer and ionic strength that isostatic, but they clearly describe that these conditions are maintained at the same levels during the course of the reaction. No particular further teaching is required for what these particular conditions should be, since they would be known to one skilled in the art. For instance reference was made in [0009] of the '926 application for systems that used Reverse Transcriptase, RNase H, primer binding steps and RNA transcription taking place simultaneously in the 3SR and NASBA systems, i.e., essentially under isostatic conditions of temperature, buffer, and ionic strength. The present invention would be able to avail itself of these conditions and with an added liberty that transcription is not required to take place during the claimed reaction, thereby avoiding restrictions on temperature, buffer, and ionic strength that may have been imposed to include suitable conditions for the transcription process.

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Applicants wish to refer also to [0017] of published U.S. 2005/0123926 where a general statement is made:

The process comprises three steps, including (a) providing a nucleic acid sample containing or suspected of containing the sequence of a specific nucleic acid; (b) contacting the sample with a three component reaction mixture; and (c) allowing the mixture to react under isostatic conditions of temperature buffer, and ionic strength, thereby producing more than one copy of the specific nucleic acid.

A variety of different methods are then described that carry out his basic function, including the embodiment that also includes RNase H and primers that comprise RNA segments. It should be pointed out that it is in the nature of isothermal reactions that although they are described in terms of discrete steps, after a mixture is formed and incubated, any and all steps start taking place contemporaneously such that at least in the mixture while some molecules may be carrying out first extension reactions, other molecules are extended primers that are being acted upon by RNase H and still others are having subsequent binding steps, and so on. As such, the entire process of the various present claims are considered to be taking place under the aforementioned isostatic conditions. Thus, the same conditions where primers are extended are the same conditions under which RNase H digestions are taking place; the same conditions that are used for a first binding of a primer are the same conditions where a new primer binds after RNase digestion regenerates a primer binding site.

With regard to page 10 and a proposed correspondence of Scheele and step (d) of the process of claim 143, Applicants again wish to point out that in Scheele amplification takes place first and then is followed by RNase H (and exonuclease) treatment (see Column 8, lines 58-68 and Column 9, lines 1-5 in Scheele). This section does not describe the use of RNase H to permit further primer binding events or synthesis of further strands of DNA. RNase H is only used by Scheele as a post-amplification procedure.

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With regard to page 12, claims 146-148 are considered to be obvious over the combination of Scheele in light of Schuster. A proposed correspondence between the claims and Scheele are presented on pages 12 and 13. First off, with regard to comments on page 12, as described above, appropriate parameters for isostatic conditions of temperature, buffer and ionic strength would be known to a person skilled in the art.

With regard to page 13, the discussion of step (d) of claim 146 being described by the adaption of PCR by Scheele has also been extensively discussed above. As pointed out, there is no use of RNase H to produce more than one copy in Scheele.

With regard to comments on page 13 concerning claim 148, there is again the mutually exclusive definitions of the relationship between the DNA target and the primer where for the purposes of being described by claim 146, the Scheele primer is considered in the Office Communication to be complementary to the DNA of interest because the DNA of interest includes the added tail and for the purposes of the being described by claim 148 (which is dependent form claim 146), the Scheele primer is not considered in the Office Action to be complementary to the DNA of interest since it is now defined as not including the added tail. Thus, the instant rejection appears to be premised upon an internally inconsistent choice of definitions and characterizations.

In view of the above claim amendments and foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the anticipation rejection.

#### **Commonality of Ownership**

Applicants assert that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made.

#### **The First Rejection Under 35 U.S.C. §103**

Claims 146-148 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Scheele (US Patent 5,162,209, issued on November 10, 1992) In view of Schuster

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et al. (US Patent 5,169,766, issued on December 8, 1992). According to the Office Communication (pages 12-14):

With regard to Claim 146, step a, Scheele teaches providing a first DNA strand (e.g. a DNA molecule of interest) (column 3 lines 25).

With regard to step b, Scheele teaches contacting the DNA with dNTPs (e.g. nucleic acid precursors) (Column 4 lines 20-22). Scheele teaches a primer comprising an RNA segment and a DNA segment by teaching that some of the nucleotide sequence of the primer is an RNA sequence such that the rest of the sequence would be DNA (e.g. a copolymer primer) (Column 3 lines 33-40). Scheele teaches a method of adding E. coli DNA polymerase I (e.g. effective amount of nucleic acid producing catalyst) (column 4 lines 14-15). Scheele teaches a method of adding RNase H (Column 4 lines 25). Therefore Scheele teaches a method of adding a polymerase and RNase H to the sample and does not teach adding a reverse transcriptase having RNase H activity.

With regard to step c, Scheele teaches a method of carrying out synthesis in the presence of the RNA primer to generate a polynucleotide comprising an RNA/DNA hybrid (Column 3 lines 30-40 and Figure 5). The instant specification does not define isostatic conditions of temperature, buffer and ionic strength. Scheele teaches combining the primer and the DNA molecule in a reagent solution at a particular heat with buffers which would have particular ionic strength to produce at least one copy of the DNA molecule by extension of the primer (Column 8 lines 1-15).

With regard to step d, Scheele teaches a method of digesting the substrate with RNase H to remove the ribonucleic acid segment of the extended primer (Column 3 lines 30-40, column 4 lines 25-28, and Figure 5). Scheele teaches that the method can be adapted to permit amplification of the sample of dsDNA by PCR methodology (column 8 lines 58-60). Scheele teaches that the reagents of the RNA primer, DNA target, dNTPs and Taq are placed into a PCR machine with the appropriate number of PCR temperature cycles (column 8 lines 65-69). Therefore in a PCR cycle the resultant dsDNA of step c would be denatured such that multiple copies of the DNA could be copied from the DNA of interest.

With regard to Claim 147, Scheele teaches that the primer can include DNA and RNA (Column 3 lines 40-45).

With regard to Claim 148, Scheele teaches that the primer includes a portion which is complementary to a portion of the oligonucleotide tail added to the 3' end of the target DNA template (column 3 lines 25-35). Therefore the primers include a sequence which is complementary to the tail and therefore noncomplementary to the sequence of the DNA

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molecule of interest (e.g. the tail is added to the DNA target of interest) (Figure 5 and column 3 lines 25-40).

Scheele et al. teaches a method of producing copies of a DNA molecule using polymerase and RNase H, however, does not teach method steps of reverse transcriptase having RNase H activity.

Schuster et al. teaches a method of amplification of nucleic acid molecules (abstract). With regard to Claim 146, Schuster et al. teaches that transcription can be done with a reverse transcriptase that has RNase H activity (column 8 lines 17-24).

Therefore it would be prima facie obvious to one of ordinary skill in the art to modify the method of Scheele et al. to replace the step of adding a polymerase and RNase H to the nucleic acid sample for a step of adding reverse transcriptase with RNase H activity as taught by Schuster et al. with a reasonable expectation of success. The ordinary artisan would be motivated to replace the step of adding a polymerase and RNase H to the nucleic acid sample for a step of adding reverse transcriptase with RNase H activity as taught by Schuster et al. because Schuster et al. teaches that if an enzyme with RNase H activity is used it is possible to omit a separate RNase H digestion step (Column 8 lines 17-24). Therefore the use of reverse transcriptase with RNase H activity would allow the ordinary artisan to perform the method of Scheele et al. with a reduced number of method steps because only reverse transcriptase with RNase activity must be added to the target to initiate transcription rather than a polymerase and RNase H and thereby allow for a quicker production of DNA molecules.

The first obviousness rejection is respectfully traversed.

The material differences between Scheele and the present invention and claims have been described in detail above. Applicants respectfully submit that the addition of Schuster et al. to Scheele's disclosure is likewise insufficient to reach their inventions defined by claims 146-148.

With regard to comments on page 14, Schuster is now being analyzed with regard to the subject claims 146-148. Although Applicants might be willing to recognize that it might be obvious to replace the polymerase used for primer extension by a reverse transcriptase, such a substitution would not reach the present invention because the only element that is conceivably added by Schuster et al. is replacement of a polymerase and a separate RNase H enzyme by a single enzyme (reverse

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transcriptase) containing both activities. Schuster et al. simply provides a minor variant of Scheele's method and, thus, the combination of the two would not have rendered Applicants' present invention obvious at the time it was made. There is no description in Schuster et al. that an extended primer made from a primer containing an RNA segment can be digested by RNase H to allow another binding and extension event. Thus, the combination of Schuster et al. with Scheele does not cure the deficiencies of the latter.

Applicants also note that step (d) of claim 146 has been amended to read "... to render said primer binding site available for another primer binding event and thereby producing more than one copy of said DNA molecule." The foregoing change is believed to clarify that the removal of the RNA is the causative agent that allows a another primer binding event that creates another copy of the DNA molecule of interest through extension of the primer after it has become bound.

In view of the foregoing remarks and the above amendment to claims 146-148, Applicants respectfully request reconsideration and withdrawal of the first obviousness rejection.

### **The Second Rejection Under 35 U.S.C. §103**

Claims 133-140 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Schuster et al. (US Patent 5,169,766, issued on December 8, 1992) in view of Vary et al. (US Patent 4,851,331, issued on July 25, 1989). According to the Office Communication (pages 15-17):

With regard to Claim 133, Schuster et al. teaches amplification of RNA (abstract). With regard to step a, Schuster et al. teaches a nucleic acid sample containing a RNA molecule of interest (Figure 2, 1st step).

With regard to step b, Schuster et al. teaches contacting the RNA molecule with nucleic acid precursors (Column 7 lines 60-65). Schuster et al. teaches annealing primers which are complementary to the RNA molecule of interest (Figure 2), however, does not teach that these primers have an at least one ribonucleic acid segment. Schuster et al. teaches addition of a nucleic acid producing catalyst (e.g. DNA

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polymerase) (Figure 2). Schuster et al. teaches the addition of RNase H (Figure 2).

With regard to step c, Schuster et al. teaches a DNA copy form the RNA molecule of interest by binding of the primer (Figure 2 step 3).

With regard to step d, Schuster et al. teaches that the first DNA copy (e.g. the cDNA) is used a template to produce a double stranded nucleic acid (e.g. double stranded DNA).

With regard to step e, Schuster et al. teaches destroying RNA with RNase H to produce the first DNA double strand copy. Schuster et al. teaches that RNA is transcribed and that the process can be continued to amplify multiple copies of the RNA molecule of interest (Figure 2).

With regard to Claim 134, Schuster et al. teaches a method wherein the primers comprise nucleotides (e.g. unmodified) (column 5 lines 55-60).

With regard to Claims 135-136, Schuster et al. teaches that the primers can comprise regions which can be used as a template for T7 RNA polymerase (column 8, lines 52-53); therefore these regions would encompass at least 1 noncomplementary nucleotides to the target.

With regard to Claims 138-139, Schuster et al. teaches the use of E. coli DNA polymerase I and Klenow polymerase (column 7 lines 15-20).

With regard to Claim 140, Schuster et al. teaches the use of Taq polymerase (column 7 lines 15).

However, Schuster et al. does not teach that the primer is comprised of RNA segments.

With regard to Claims 133 and 137, Vary et al. teaches that when using a primer-dependent DNA polymerase of eukaryotic origin primers having a 3' terminal ribonucleotide rather having a 3' terminal deoxynucleotide are more active (Column 9 lines 65-69 and column 10 lines 1-10). Vary et al. teaches that oligonucleotides can be comprised of both RNA and DNA (column 10 lines 5-10). The polymerase of Schuster et al. is E. coli DNA polymerase I which is a polymerase of eukaryotic origin.

It would have been prima facie obvious to one of ordinary skill in the art at the time of filing to modify the method of Schuster et al. to use a RNA/DNA primer as taught by Vary et al. in place of the DNA primer used to transcribe the RNA to cDNA. The ordinary artisan would be motivated to use a DNA primer with an end of ribonucleotide in order to have a more active elongation of the template region using E. coli DNA polymerase I. Vary et al. teaches that when using a primer-dependent DNA polymerase of eukaryotic origin primers having a 3' terminal ribonucleotide rather having a 3' terminal deoxynucleotide are more active (Column 9 lines 65-69 and column 10 lines 1-10).

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The second obviousness rejection is respectfully traversed.

With reference to claims 133-140, the Office Communication posits Vary et al in combination with Schule. Neither patent describes, however, the removal of RNA segments of an extended primer to generate a primer binding site. Schuster et al. describe digestion of RNA transcripts (nucleic acids that have been generated without a primer) and have no description of digestion of extended primers. The Schuster patent merely contains the standard method of eliminating the original RNA transcript after it has been used as a template to synthesize a cDNA molecule in preparation for conversion of the cDNA into a double-stranded DNA molecule.

Step (e) of the process of claim 133 describes the nuclease digestion of the extended primer after conversion of the first extended primer ("the first DNA copy") into double stranded form in step (d). There is no description in Schuster et al. for treating a double-stranded DNA molecule [the product of step (d)] with an RNase H as is required to take place in step (e) in present claim 133.

With regard to Vary et al., no benefit for a primer binding event is realized after excision of a nucleotide as described in their patent. The only benefit that is conveyed by the passages cited from the Vary et al. is that the use of ribonucleotide in the 3' end can offer superior extension characteristics when a eukaryotic derived polymerase is used. No particular advantages are described or suggested to be endowed by the presence of one or more ribonucleotide moieties after it has been extended in Vary et al. In sharp contrast, the present method and claims enjoys its properties by elimination of ribonucleotides after an extension of a primer with an RNA segment has taken place.

In sum, the combination of Schuster et al. in view of Vary et al. would not have rendered the present invention and claims obvious to the ordinarily skilled person in the art.

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Reconsideration and withdrawal of the second obviousness rejection is respectfully requested.

**Information Disclosure Statement**

Concurrently with their Amendment, Applicants are submitting their Information Disclosure Statement as evidenced by two sheets of listed documents on Form PTO/SB/08a (01-08) [1 sheet] and Form PTO/SB/08b (01-08) [1 sheet]. Also being submitted are three (3) non-U.S. patent documents listed on the PTO Form PTO/SB/08b (01-08).

Applicants are submitting this IDS in order to bring to the attention of the Patent Office and the Examiner a number of documents including the three (3) non-U.S. patent documents.

It is respectfully requested that these three documents and the other documents listed in Applicants' IDS be made of record in this application and considered by the Examiner in determining whether the present claims are patentable.

Early and favorable action is respectfully requested.

\* \* \* \* \*

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### **SUMMARY AND CONCLUSIONS**

In the above claim listing for this application, claims 112, 143 and 146 have been amended; claims 141-142 have been canceled; and new claims 149-150 have been added. No other claims have amended, canceled or added by this paper.

No claim fee is believed due for this paper because the same number of claims is being presented than the number of previously paid for claims. This paper is also accompanied by a Request For Extension Of Time (3 months) and Applicants' Information Disclosure Statement and authorization for the fees therefor. No other fee or fees are believed due in connection with this paper or the accompanying papers, the extension request and the IDS. In the event that any other fee(s) is/are due in connection with this filing, however, the Patent and Trademark Office is hereby authorized to charge the amount of any such fee(s) to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone call would be helpful in the processing of this paper or this application, Applicants' undersigned attorney requests that he be contacted at the numbers below.

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Appendix A To Applicants' September 30, 2009 Amendment Under 37 C.F.R. §1.115  
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# APPENDIX A

**Enz-52(D2)(C)(D1)**



## MINIREVIEW

**Ribonuclease H: properties, substrate specificity and roles in retroviral reverse transcription**

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**Keywords**

catalytic mechanism; DNA/RNA hybrids; endonuclease; human immunodeficiency virus, type 1; Moloney murine leukemia virus; polypurine tract; reverse transcriptase; reverse transcription; RNA cleavage; RNase H

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Retroviral reverse transcriptases possess both a DNA polymerase and an RNase H activity. The linkage with the DNA polymerase activity endows the retroviral RNases H with unique properties not found in the cellular counterparts. In addition to the typical endonuclease activity on a DNA/RNA hybrid, cleavage by the retroviral enzymes is also directed by both DNA 3' recessed and RNA 5' recessed ends, and by certain nucleotide sequence preferences in the vicinity of the cleavage site. This spectrum of specificities enables retroviral RNases H to carry out a series of cleavage reactions during reverse transcription that degrade the viral RNA genome after minus-strand synthesis, precisely generate the primer for the initiation of plus strands, facilitate the initiation of plus-strand synthesis and remove both plus- and minus-strand primers after they have been extended.

At the time of its discovery in 1970, the presence of an RNA-dependent DNA polymerase activity in retrovirus particles provided strong and exciting support for the hypothesis that the single-stranded RNA genome of a retrovirus is replicated through a DNA intermediate [1,2]. Not only did this discovery of reverse transcriptase (as it was dubbed) challenge the existing dogma concerning the flow of genetic information in biology, it raised the critical question as to how the DNA/RNA hybrid created when the viral genome RNA is used as a template by reverse transcriptase is further processed. In retrospect, it is not surprising that an RNase H activity that degrades the RNA strand of a DNA/RNA hybrid is required to free the newly made DNA strand (called the minus strand because it is complementary to the plus genome RNA) for use as a template in the synthesis of the second or

plus strand DNA. However, it was a surprise when the retroviral-specific RNase H activity turned out to be present in the same protein molecule as the polymerase activity [3]. This intimate association of the DNA polymerase and RNase H activities in reverse transcriptase has profound effects on the activities and capabilities of both enzymes.

This minireview provides a summary of the salient features of retroviral RNases H with a focus on how the shared substrate-binding sites for the two activities of reverse transcriptase endow the retroviral RNases H with features not found in the cellular counterparts, and how these unusual properties are crucial for the multiple roles played by RNase H in reverse transcription. Although occasional reference is made to other retroviral enzymes, the primary focus is on the well-studied RNase H activities associated with human

**Abbreviations**

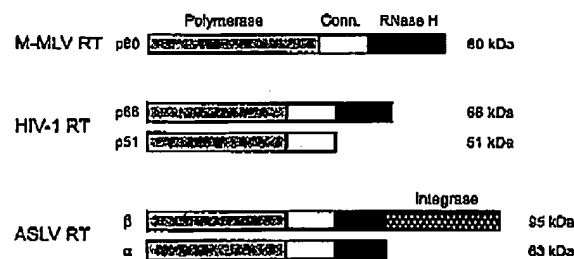
HIV-1, human immunodeficiency virus, type 1; M-MLV, Moloney murine leukemia virus; PBS, primer binding site; PPT, polypurine tract.

immunodeficiency virus, type 1 (HIV-1) and Moloney murine leukemia virus (M-MLV) reverse transcriptases. The reader is directed to other excellent reviews that describe the older literature and cover other recent aspects of retroviral RNases H [4–8].

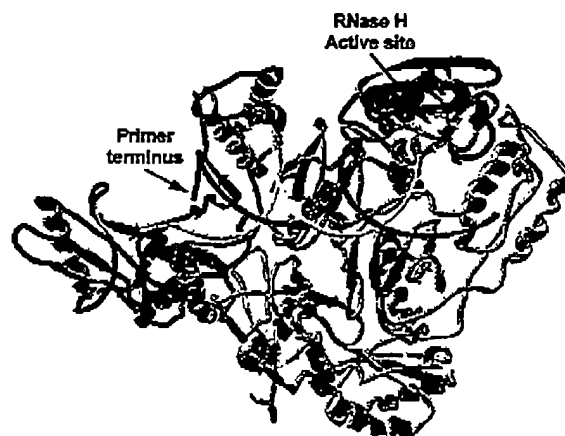
### Structure–function considerations

Although the reverse transcriptases from murine, human and avian retroviruses display different subunit structures, the relative orientations and sizes of the DNA polymerase, connection and RNase H domains within a given polypeptide chain are similar for the different proteins (Fig. 1). M-MLV reverse transcriptase is an 80 kDa monomer in which the DNA polymerase activity occupies the N-terminal ~55% and the RNase H domain occupies the C-terminal ~25% of the protein, with the connection domain accounting for the remainder. HIV-1 reverse transcriptase is a heterodimer made up of a p66 subunit containing the active forms of both the polymerase and the RNase H arranged similarly to that of the M-MLV monomer, and a p51 subunit that is derived by proteolysis of p66 and is missing the C-terminal RNase H domain (Fig. 2). The p51 subunit is enzymatically inactive and simply plays a structural role in the protein. The avian sarcoma-leukosis virus (ASLV) reverse transcriptase is also a heterodimer, but the larger  $\beta$  subunit, in addition to possessing both the polymerase and RNase H domains found in the  $\alpha$  subunit, also contains a C-terminal region corresponding to the viral integrase.

The isolated RNase H domain of M-MLV reverse transcriptase is enzymatically active, but the activity is



**Fig. 1.** Subunit and domain structures of retroviral reverse transcriptases. Reverse transcriptase from M-MLV is a monomer, whereas the HIV-1 and avian sarcoma-leukosis virus (ASLV) reverse transcriptases are both heterodimeric. The subunit designations and their sizes (kDa) are indicated along the left and right sides of the figure, respectively. The approximate sizes of the polymerase, connection (conn.) and RNase H domains are shown in gray, white, and black, respectively. The larger  $\beta$  subunit of the avian sarcoma-leukosis virus reverse transcriptase also contains the integrase domain depicted by cross-hatching.



**Fig. 2.** Ribbon diagram of the co-crystal structure of HIV-1 reverse transcriptase with a bound RNA template and DNA primer (PDB entry 1HYS) [19]. The polymerase (p66 residues 1–318), connection (p66 residues 319–437) and RNase H (p66 residues 438–553) domains are drawn in red, green and blue, respectively with the p51 subunit shown in gray. The RNase H active site is indicated with the four key acidic residues drawn in yellow ball and stick. The primer terminus of the DNA primer strand (purple) is indicated with the RNA template strand shown in yellow. The drawing was created using SWISS-PDB VIEWER software (v. 3.7) (GlaxoSmithKline, Brentford, UK).

low and exhibits a greatly relaxed substrate specificity [9–11]. The isolated HIV-1 RNase H domain is inactive, but the addition of various N-terminal extensions restores some RNase H activity [12–18]. The reduced specificity of the isolated RNase H domains underscores the importance of the polymerase and connection domains for substrate binding and selectivity. Structural models support this conclusion by showing that a DNA/RNA hybrid substrate gains access to the RNase H active site by associating with the same binding cleft utilized by the polymerase for binding a primer-template [19] (Fig. 2). Some of the structural features within and outside the RNase H domain that are important for substrate selectivity are highlighted in the remainder of this section.

The polymerase domain has been directly implicated in RNase H specificity through the mutagenesis of individual amino acids. Notable examples include changes at Trp266 and Phe61 in HIV-1 reverse transcriptase, both of which render the RNase H incapable of generating the polypurine tract (PPT) primer or removing the PPT primer once it has been extended [20–22].

The RNase H domains of M-MLV and HIV-1 reverse transcriptases are structurally very similar to the *Escherichia coli* and *Bacillus halodurans* RNases H, and to human RNase H1, and these similarities



provide key insights concerning substrate recognition and catalysis by the retroviral enzymes. One conspicuous difference among these enzymes is a positively charged helix called the C-helix that is present in the M-MLV, human and *E. coli* RNases H, but absent in the RNases H from HIV-1 and *B. halodurans* [19,23–30]. Structure-function studies with the *E. coli* and M-MLV RNases H implicate the C-helix in substrate recognition and catalytic activity, and a mutant form of the M-MLV reverse transcriptase in which the C-helix has been deleted is replication defective [31–33]. Despite the apparent absence of a C-helix in the RNase H domain of HIV-1 reverse transcriptase, modeling studies comparing the C-helix of M-MLV RNase H with the p66 subunit of the HIV-1 enzyme suggest that a series of positively charged residues in the p66 connection domain may functionally substitute for the C-helix in the HIV-1 reverse transcriptase [34]. Mutagenesis studies with HIV-1 reverse transcriptase identify additional residues within the connection domain that contribute to the activity of the RNase H [35] and linker scanning mutagenesis of the M-MLV connection domain indicate that this region is essential for viability of the virus [36].

The RNase H primer grip is a region near the RNase H active site that contacts the nucleotides in the DNA strand of the hybrid substrate that are base paired with RNA nucleotides at positions –4 to –9 relative to the site of cleavage, which is defined as occurring between the –1 and +1 RNA nucleotides [19,34]. For HIV-1 reverse transcriptase, this region includes residues found in the polymerase, RNase H and connection domains of p66, and also two residues present in the p51 subunit. The RNase H primer grip is important for binding the DNA/RNA hybrid substrate because point mutations in this region not only reduce RNase H activity, but also affect the specificity of the enzyme [35,37–40]. Primer grip residue Tyr501 in HIV-1 reverse transcriptase (Tyr586 in M-MLV) appears to be a particularly important substrate contact residue because changes at this site profoundly affect both the RNase H activity and proper substrate recognition [37,39–42]. Gln475 in HIV-1 reverse transcriptase is also a critical primer grip residue that not only interacts with the DNA strand, but also contacts the RNA strand at positions –2 and +1. Mutagenesis studies indicate that Gln475 is particularly important for the cleavage specificity of the enzyme [39].

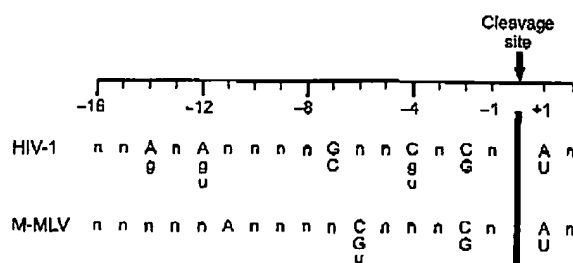
Based on co-crystal structures of HIV-1 reverse transcriptase with DNA duplexes or DNA/RNA hybrids [19,25,27], the physical distance between the 3'-end of a primer located in the polymerase active site

and the region of the substrate in close contact with the RNase H active site corresponds to 17–18 bp (Fig. 2). This relationship helps explain some of the observations concerning the effects of recessed DNA 3'- and RNA 5'-ends on RNase H specificity as described in the sections to follow.

### Enzyme activity and catalysis

Retroviral RNases H are partially processive endonucleases that cleave the RNA strand of a DNA/RNA hybrid in a  $Mg^{2+}$ -dependent reaction to produce 5' phosphate and 3' hydroxyl termini [43,44]. It has been shown that the RNases H associated with both HIV-1 and M-MLV reverse transcriptases are capable of cleaving RNA/RNA duplexes, an activity that has been termed RNase H\* [45–47]. However, because the RNase H\* activity is only manifest in the presence of the less biologically relevant divalent cation,  $Mn^{2+}$ , it is doubtful that this activity plays a role during reverse transcription *in vivo*. Given a substrate in which one strand is entirely DNA and the other strand is RNA at the 5'-end followed by a stretch of DNA, the HIV-1 and M-MLV retroviral RNases H strongly prefer to cleave the RNA strand one nucleotide away from the RNA–DNA junction rather than precisely at the junction itself [48]. As discussed later, the most dramatic example of this preference is the finding that a single ribo A is left on the 5'-end of the DNA during tRNA primer removal by HIV-1 RNase H [14,49,50]. However, this preference to cleave one nucleotide away from the RNA–DNA junction is not absolute because in the presence of other specificity determinants, the retroviral RNases H will cleave precisely at an RNA–DNA junction [49,51,52].

Two recent co-crystal structures of the *B. halodurans* and human RNases H with bound substrate [29,30,53,54] provide key insights into the role of divalent cations in the catalytic mechanism of the structurally similar RNase H domains of HIV-1 and M-MLV reverse transcriptases. Thus, the current model for hydrolytic cleavage by the retroviral RNases H invokes a two- $Mg^{2+}$ -ion catalytic mechanism [8]. In HIV-1 RNase H, four highly conserved acidic amino acids (Asp443, Glu478, Asp498 and Asp549) coordinate the binding of two  $Mg^{2+}$  ions. The corresponding active site amino acids in the M-MLV enzyme are Asp524, Glu562, Asp583 and Asp653. Catalysis involves activation of the nucleophilic water by one of the  $Mg^{2+}$  ions, with transition-state stabilization apparently being achieved by both  $Mg^{2+}$  ions.



**Fig. 3.** Sequence preferences for internal cleavage by retroviral RNases H. For the purposes of site alignment, RNase H cleavage is designated as occurring between nucleotides -1 and +1. The preferred nucleotides at positions -14, -12, -7, -4, -2 and +1 are shown for HIV-1 RNase H and at positions -11, -6, -2 and +1 for M-MLV RNase H. The strongest preferences are indicated in upper case letters with the weaker preferences in lower case letters.

### Substrate specificity

Three distinct cleavage modes have been described for retroviral RNases H that are referred to as internal, DNA 3'-end-directed and RNA 5'-end-directed cleavages. The two end-directed modes are unique to the retroviral RNases H and derive from the presence of the associated polymerase domain. In the internal cleavage mode, the RNases H behave as typical endonucleases and cleave the RNA along the length of a DNA/RNA hybrid substrate in the absence of any 'end' effects. In the two end-directed modes of cleavage, the interaction of the enzyme with the substrate involves recognition of a recessed RNA 5'- or a recessed DNA 3'-end.

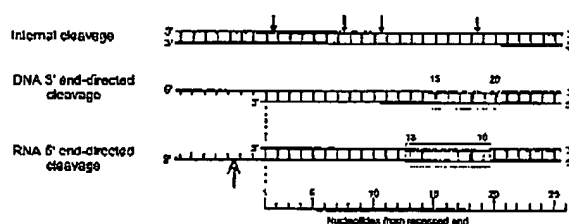
### Internal cleavage

Although cleavage at internal sites on an extended DNA/RNA hybrid has been inferred from a variety of studies over the years, only recently has it been recognized that nucleotide sequence preferences play an important role in this mode of cleavage. HIV-1 and M-MLV RNase H cleavage sites that were too far from an end to be either DNA 3'- or RNA 5'-end-directed were mapped on a long DNA/RNA hybrid and the nucleotide sequences surrounding the scissile phosphate (designated as between the -1 and +1 positions) were aligned. Statistical analysis of the frequency of nucleotides on both sides of the cleavage site revealed that HIV-1 RNase H prefers certain nucleotides at positions +1, -2, -4, -7, -12 and -14. For M-MLV, the preferred positions are located at +1, -2, -6 and -11 (Fig. 3) [8,55]. Notably, the preferred nucleotides at the +1 (A or U) and -2 (C or G) positions are the same for the two enzymes. The preferred positions all fall within a region of the substrate

contacted by the enzymes as defined by the co-crystal structure containing a DNA/RNA hybrid [19] and by DNase I footprinting studies [56–58]. The structural basis for these sequence preferences remains for the most part obscure, but the contact between Gln475 in the HIV-1 enzyme and the -2 guanine base in the RNA strand likely contributes to the preference at this position [19].

### DNA 3'-end-directed cleavage

A recessed DNA 3'-end in a DNA/RNA hybrid is recognized by the polymerase activity of reverse transcriptase as a primer terminus and is utilized for the synthesis of a DNA strand complementary to the RNA. In the absence of dNTPs or at a pause site during polymerization, the active site of the RNase H activity would be predicted, based on structural models, to be positioned 17–18 nucleotides away from the DNA primer terminus (Fig. 4) [19,25,27]. Results from a number of laboratories indicate that RNase H cleavage of a hybrid with a recessed DNA 3'-end, or at pause sites during polymerization, actually occurs within a window ~15–20 nucleotides away from the primer terminus (Fig. 4) [59–64]. Notably, the cleavage window centers on the distance predicted from the crystal structures, but extends in both directions by 2–3 bp, presumably owing to some degree of structural variation in the substrate and flexibility within the protein.



**Fig. 4.** Three cleavage modes for retroviral RNases H. DNA/RNA hybrids are drawn with RNA strands in red and DNA strands in black. In the internal cleavage mode, the arrows mark the sites of cleavage along the length of the hybrid where nucleotide sequence alone determines the cleavage site. The cleavage window for the DNA 3'-end-directed cleavage mode (15–20 nucleotides from the recessed DNA end) is highlighted in green. The corresponding cleavage window for RNA 5'-end-directed cleavage (13–19 nucleotides from end) is highlighted in blue. The open-headed arrow in the RNA 5'-end-directed cleavage mode indicates the position of the DNA phosphate that appears to be bound near the active site pocket in the polymerase domain normally occupied by the 3' DNA primer terminus during DNA polymerization.

### RNA 5'-end-directed cleavage

Unexpectedly, reverse transcriptase will bind to a hybrid duplex containing a recessed RNA 5'-end and cleave the RNA ~13–19 nucleotides from the RNA end (Fig. 4) [60,65–72]. RNase H cleavage only occurs at sites within the window that conform to the nucleotide sequence preferences for internal cleavage that are proximal to the active site of the enzyme [72]. It is not known why the window for RNA 5'-end-directed cleavage is two nucleotides closer to the recessed end than the window for DNA 3'-end-directed cleavage. However, based on this difference, a phosphate residue in the single-stranded DNA that extends two nucleotides beyond the recessed RNA 5'-end (Fig. 4, open-headed arrow) would be predicted to occupy the position in the polymerase active site normally occupied by the primer terminus during DNA synthesis. Presumably some feature of the polymerase active site region interacts with the recessed RNA 5'-end to facilitate

this unique binding configuration to the primer-template binding cleft of reverse transcriptase.

In some studies, cleavage in the RNA 5'-end-directed mode has been observed as close as 7 bp and as many as 21 bp from the recessed end [67,73–78], possibly resulting from sliding of the enzyme after the initial binding event. Importantly, an RNA 5'-end at a nick is not recognized for this mode of cleavage by the HIV-1 and M-MLV RNases H. However, cleavage will occur by this mode if a gap of 2–3 nucleotides is present upstream of the RNA 5'-end.

### Roles of RNase H in reverse transcription

Starting with the retroviral plus-strand genome, the process of reverse transcription produces a double-stranded DNA product that is integrated into the host cell genome and ultimately serves as a template for the production of more genome RNAs [79,80]. The RNase H activity of reverse transcriptase is required

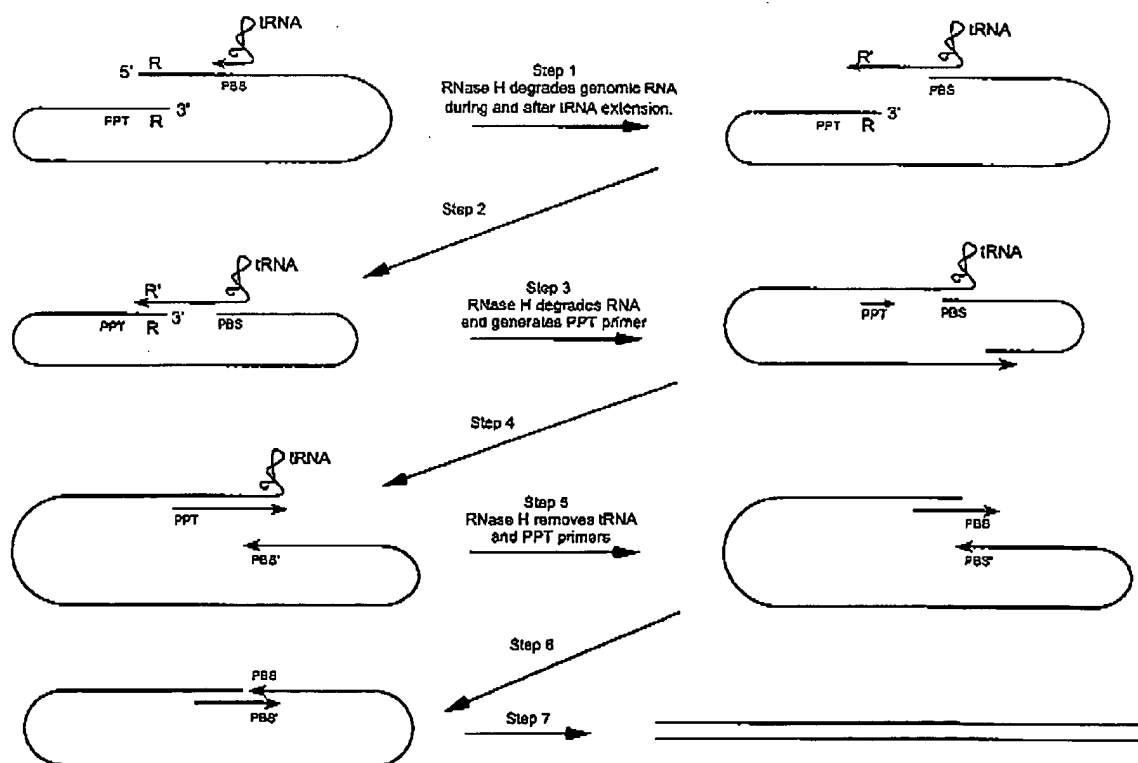


Fig. 5. Roles of RNase H in reverse transcription. The retroviral genome and the associated cell-derived tRNA bound to the PBS are shown in red with the DNA strands produced during reverse transcription shown in black. A repeated sequence denoted R is located at both ends of the retroviral genome. The sequences complementary to PBS and R are denoted PBS' and R', respectively. The PPT serves as the primer for plus-strand synthesis. The steps at which RNase H plays a role are highlighted. See the text for a detailed explanation.

for several stages of the reverse transcription process [4,6,7], making it an essential enzyme activity for viral replication [32,81]. Although all retroviruses have diploid genomes and template switching between genomes has been observed during reverse transcription, only a single genome strand is considered in the following discussion. The key steps in M-MLV and HIV-1 reverse transcription are summarized below with an emphasis on the multiple roles played by RNase H in the process (Fig. 5).

### Step 1

Early after infection a subviral particle enters the cytoplasm containing, in addition to the viral RNA associated with the nucleocapsid protein, a host-derived tRNA bound to the genome at the 18 nucleotide-long primer binding site (PBS), 50–100 molecules of reverse transcriptase and the integrase. As shown in Fig. 5, the polymerase activity of reverse transcriptase initiates reverse transcription by extending the tRNA primer to copy the 5' repeat sequence (R) at the end of the genome and produce what is called the minus strong-stop DNA. Concomitant with polymerization and presumably at pause sites [63,64], the RNase H activity utilizes the DNA 3'-end-directed cleavage mode to cleave the RNA strand of the resulting hybrid. However, for HIV-1 and M-MLV reverse transcriptases, such cleavages occur on average only once for every 100–120 nucleotides polymerized, a frequency that is insufficient to degrade the RNA into small enough fragments to render the newly synthesized DNA free of RNA [62,82]. Therefore, complete degradation of the template RNA likely requires multiple internal cleavages to generate gaps that subsequently enable degradation by the RNA 5'-end-directed mode of cleavage.

### Step 2

When the polymerase reaches the end of the RNA template, the RNase H cleavages nearest to the 5'-end of the RNA would be expected to be determined by the cleavage window for whichever end-directed cleavage mode applies to a blunt-ended substrate. In either case, a short RNA oligonucleotide would likely remain base paired with the 3'-end of the nascent DNA chain. In fact, for HIV-1, it has been observed that in the presence of the nucleocapsid protein, a 14 nucleotide-long RNA remains associated with the DNA (not shown in Fig. 5) and, importantly this association prevents self-priming caused by the DNA hairpin (the complement of the RNA TAR structure) that

otherwise could form at the 3'-end of the nascent DNA [83,84]. Because this residual RNA fragment is short relative to the R sequence (R is 98 nucleotides for HIV-1 and 68 nucleotides for M-MLV), it does not interfere with the first template switch mediated by base pairing between the R' sequence found at the 3'-end of the minus strong-stop DNA and the R sequence found at the 3'-end of the genome RNA. Once these complementary sequences pair, branch migration displaces the short RNA oligonucleotide, positioning the 3'-end of the nascent DNA to act as a primer for the completion of minus strand synthesis.

### Step 3

The first template switch enables continued synthesis of the minus-strand DNA (Fig. 5). RNase H degradation of the genome RNA follows the same pattern as described above, beginning with the occasional DNA 3'-end-directed cleavage during polymerization, followed by sequential internal and RNA 5'-end-directed cleavages. It is likely that some longer RNA fragments remain base-paired to the minus DNA and must be removed by displacement synthesis during polymerization of the plus-strand DNA [82,85].

Once the PPT region of the genome has been copied, a specific RNase H cleavage near the 3'-end of the polypurine sequence generates the primer for plus-strand initiation [8]. Underscoring the importance of this specific cleavage event is the fact that the initiation site of the plus-strand DNA determines the left end of the linear product of reverse transcription (Fig. 5) which is a substrate for the viral integrase. Although cleavage at the PPT site is very efficient in the internal cleavage mode for M-MLV, HIV-1 reverse transcriptase is less efficient in this mode and cleavage may instead occur through the DNA 3'-end-directed mode at a pause site during HIV-1 minus-strand synthesis [21,48,52,66,86–93]. A possible explanation for the reduced efficiency of cleavage by the HIV-1 enzyme is that although the M-MLV PPT sequence conforms to the preferred nucleotide pattern for internal cleavage described above (Fig. 3), there is an A instead of the preferred G or C at the –7 position of the HIV-1 PPT sequence. A variety of studies have identified the nucleotide positions within the PPT that are critical for proper cleavage and although some of these overlap with the more general preferences for internal cleavage, other positions do not. Thus, for proper PPT primer generation by M-MLV RNase H, positions –1, –2, –4, –5, –6, –7, –10 and –11 are important [51,94,95], whereas positions +1, –2, –4, –5 and –7

have been found to be critical for HIV-1 PPT primer formation [38,40,89,96–98].

#### Step 4

The PPT primer is utilized to initiate plus-strand synthesis which then continues until it reaches the 18th nucleotide in the tRNA where further synthesis is blocked by a methylated base (Fig. 5). This product has been referred to as plus strong-stop DNA. At least for M-MLV, a nick within the PPT that generates the correct primer terminus for plus-strand initiation is poorly utilized in the displacement synthesis mode by the polymerase activity of reverse transcriptase [99]. Efficient utilization of the PPT primer requires at least a small gap and indeed there exists a series of internal RNase H cleavage sites just downstream of the PPT that would appear to fulfill this role.

#### Step 5

Continued synthesis of the minus and plus strands requires removal of the extended tRNA primer from the end of the minus DNA (Fig. 5). With further extension temporarily blocked by a methylated base at position 19 in the tRNA, the tRNA–DNA junction is within the 15–20 nucleotide window required for DNA 3′-end-directed cleavage. As mentioned previously, the RNase H activity of reverse transcriptases strongly prefers to cleave one nucleotide away from an RNA–DNA junction and indeed for HIV-1, tRNA primer removal is observed to cleave the RNA between the 17th and 18th nucleotides from the nascent DNA 3′-end to leave a single ribo A on the 5′-end of the minus-DNA strand [14,49,50]. Furthermore, cleavage precisely at the RNA–DNA junction by the HIV-1 enzyme, although still within the cleavage window, would appear to be disfavored by the presence of a dC residue at the +1 position rather than the preferred A or U. For M-MLV, cleavage to leave a single ribo A as well as junctional cleavage are both observed, presumably owing to the presence of favored nucleotides at the critical positions flanking both cleavage sites [11,100].

Removal of the PPT primer appears to occur by an internal cleavage event precisely at the RNA–DNA junction [48,51,52,90,91,101]. Apparently the same sequence features responsible for PPT primer generation determine the site of primer removal and override the natural tendency of the RNase H to cleave one ribonucleotide away from an RNA–DNA junction.

#### Steps 6 and 7

Once the tRNA primer has been removed, the second template switch is effected by the pairing of the complementary PBS and PBS′ sequences. A combination of nondisplacement and displacement synthesis [102] converts the circular intermediate into the final linear product of reverse transcription (Fig. 5).

#### Perspectives

The specificity determinants for the RNase H activities associated with retroviral reverse transcriptases derive not just from the RNase H domain itself, but also from the polymerase and connection domains. These determinants endow the enzymes with the ability to cleave DNA/RNA hybrids in the three cleavage modes described above. During reverse transcription, these specificities enable the RNase H to carry out a remarkable series of diverse cleavage reactions that lead to the degradation of the genome RNA after minus-strand synthesis, the precise generation of the PPT primer, the facilitation of plus-strand initiation, and the removal of both primers after they have been extended.

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